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Expression of Phosphatidylserine-Specific Phospholipase A₁ mRNA in Human THP-1-Derived Macrophages

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The expression of phosphatidylserine-specific phospholipase A₁ (PS-PLA₁) is most upregulated in the genes of peripheral blood cells from chronic rejection model rats bearing long-term surviving cardiac allografts. The expression profile of PS-PLA₁ in peripheral blood cells responsible for the immune response may indicate a possible biological marker for rejection episodes. In this study, PS-PLA₁ mRNA expression was examined in human THP-1-derived macrophages. The effects of several immunosuppressive agents on this expression were also examined in in vitro experiments. A real-time RT-PCR analysis revealed that PS-PLA₁ mRNA expression was found in human THP-1-derived macrophages. This expression was enhanced in the cells stimulated with lipopolysaccharide (LPS), a toll-like receptor (TLR) 4 ligand. Other TLR ligands (TLR2, 3, 5, 7, and 9) did not show a significant induction of PS-PLA₁ mRNA. The time course of the mRNA expression profiles was different between PS-PLA₁ and tumor necrosis factor- α (TNF- α), which showed a maximal expression at 12 and 1 h after LPS stimulation, respectively. Among the observed immunosuppressive agents, corticosteroids, prednisolone, 6 α -methylprednisolone, dexamethasone, and beclomethasone inhibited PS-PLA₁ expression with half-maximal inhibitory concentrations less than 3.0 nM, while methotrexate, cyclosporine A, tacrolimus, 6-mercaptopurine, and mycophenolic acid showed either a weak or moderate inhibition. These results suggest that the expression of PS-PLA₁ mRNA in THP-1-derived macrophages is activated via TLR4 and it is inhibited by corticosteroids, which are used at high dosages to suppress chronic allograft rejection.

Key words: Phosphatidylserine-specific phospholipase A₁ (PS-PLA₁); THP-1-derived macrophages; mRNA expression; Corticosteroids; Chronic rejection

INTRODUCTION

The control of chronic rejection is important for long-term graft survival in organ transplantation, because this is a significant cause of graft loss during the first year following transplantation (11). Therefore, specific and sensitive markers predicting the chronic rejection are required for improving the clinical outcome. The current study used a high-throughput microarray analysis to demonstrate that phosphatidylserine-specific phospholipase A₁ (PS-PLA₁) showed the highest upregulated expression among all the observed genes in peripheral blood cells from chronic rejection model rats with long-term surviving cardiac allografts (12).

PS-PLA₁, an enzyme of the lipase family, acts specifically on phosphatidylserine (PS) and hydrolyzes fatty acid from the *sn*-1 position of PS to produce lysophosphatidylserine (lysoPS) and fatty acid (6,7,16,20,24). Because both PS and lysoPS are associated with the activation of immune cells, including macrophages via PS and lysoPS receptors (15,18,19,21), the activity of PS-PLA₁ might be important for the immune response in regulating the production or elimination of PS and lysoPS (1,2). A marked elevation of the PS-PLA₁ expression is observed in rats treated with lipopolysaccharide (LPS) (4), thus suggesting that PS-PLA₁ plays a role in the activated immune system (1), which is associated with chronic rejection (11). The current study focused

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on the expression profile of PS-PLA₁ in immune cells as sample specimens for clinical application.

This study examined PS-PLA₁ mRNA expression in human THP-1-derived macrophages stimulated by toll-like receptor (TLR) ligands, including LPS. The effects of immunosuppressive agents on the expression were also examined using this in vitro system.

MATERIALS AND METHODS

Materials and Reagent Solutions

LPS (ultra-pure lipopolysaccharide, from *Escherichia coli* K12), heat-killed *Listeria monocytogenes* (HKLM),

polyinosine-polycytidylic acid [poly(I:C)], flagellin (from *B. subtilis*), loxoribine and oligonucleotide 2006 (ODN2006) were purchased from InvivoGen (San Diego, CA) and prepared according to the manufacturer's instructions. RPMI-1640 medium, dimethyl sulfoxide (DMSO), prednisolone (PSL), 6 α -methylprednisolone (MPSL), 6-mercaptopurine monohydrate (6MP), tacrolimus (TAC), dexamethasone (DEX), and mycophenolic acid (MPA) were purchased from Sigma-Aldrich (St. Louis, MO). Beclomethasone dipropionate (BDP) was purchased from MP Biomedicals (Solon, OH). Methotrexate (MTX), cyclosporine A (CYA), and phorbol 12-myris-

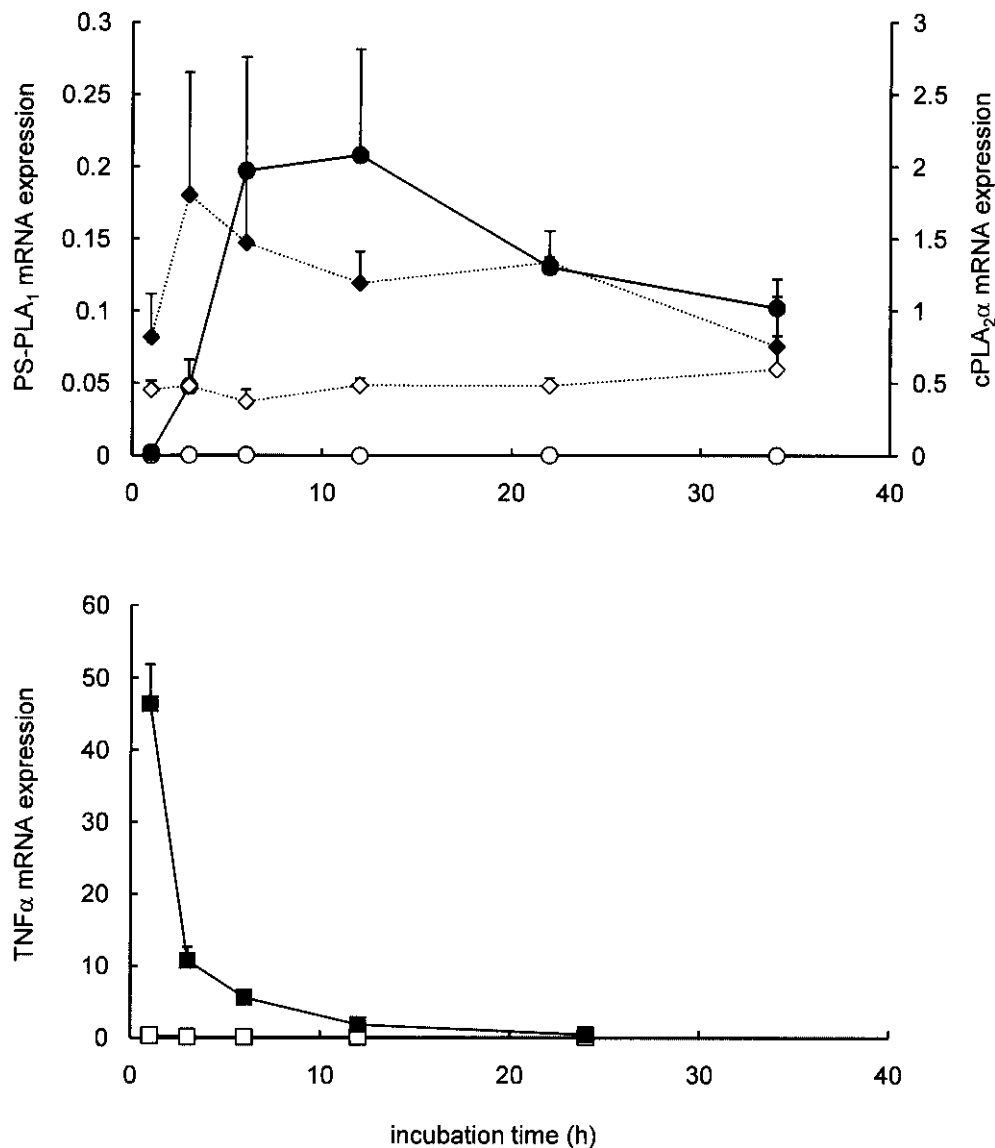


Figure 1. Time course profile of the LPS-mediated upregulation of mRNA expression for PS-PLA₁, cPLA₂α, and TNF-α in THP-1 macrophages. Differentiated THP-1 cells were incubated for 1–34 h with/without 10⁻⁵ g/ml of LPS. The relative mRNA levels of PS-PLA₁ (upper panel, left axis: with LPS, filled circles; without LPS, open circles), cPLA₂α (upper panel, right axis: with LPS, filled diamonds; without LPS, open diamonds) and TNF-α (lower panel: with LPS, filled squares; without LPS, open squares) to GUS were determined respectively by quantitative real-time RT-PCR. The results are shown as the mean ± SD of three independent experiments.

Table 1. Effects of Immunosuppressive Agents on mRNA Expression

| Stimuli | mRNA Expression Versus GUS ($\times 10$, Mean \pm SD) | |
|------------|--------------------------------------------------------------|----------------------------|
| | PS-PLA ₁ | cPLA ₂ α |
| None | 0.00 (detected)* | 4.86 \pm 0.49 |
| LPS | 2.08 \pm 0.73 | 11.90 \pm 2.18 |
| LPS + PSL | 0.09 \pm 0.02† | 1.68 \pm 0.34† |
| LPS + MPSL | 0.06 \pm 0.01† | 1.37 \pm 0.29† |
| LPS + DEX | 0.03 \pm 0.01† | 0.98 \pm 0.15† |
| LPS + BDP | 0.15 \pm 0.10† | 1.86 \pm 0.27† |
| LPS + MTX | 0.97 \pm 0.20† | 5.04 \pm 0.70† |
| LPS + 6MP | 1.51 \pm 0.39 | 6.09 \pm 2.35† |
| LPS + CYA | 1.49 \pm 0.33 | 9.00 \pm 1.21† |
| LPS + TAC | 1.94 \pm 0.53 | 7.67 \pm 0.85† |
| LPS + MPA | 2.04 \pm 0.37 | 9.48 \pm 0.20‡ |

LPS, lipopolysaccharide; PSL, prednisolone; MPSL, 6 α -methylprednisolone; DEX, dexamethasone; BDP, beclomethasone dipropionate; MTX, Methotrexate; 6MP, 6-mercaptopurine monohydrate; CYA, cyclosporine A; TAC, tacrolimus; MPA, mycophenolic acid.

*Detected mean value was 0.000840.

† $p < 0.01$ versus LPS.

‡ $p < 0.05$ versus LPS.

tate 13-acetate (PMA) were purchased from Wako Chemicals (Osaka, Japan). Fetal bovine serum (FBS) was purchased from JRH Biosciences (Lenexa, KS). All other reagents and solvents for the assay were of either analytical or reagent grade and they were purchased from Wako Chemicals or Sigma-Aldrich.

Cell Culture

The THP-1 human monocytic cell line (ATCC, TIB-202) was maintained in RPMI-1640 medium supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin at 37°C in 5% CO₂. Differentiated THP-1 cells were obtained by treatment with 5 nM PMA for 2 days and then were starved overnight in RPMI-1640 medium containing 0.5% FBS and 5 nM PMA. They were thereafter stimulated with 0–10⁻⁵ g/ml LPS in the presence of 10% heat-inactivated FBS for up to 34 h. In some experiments, differentiated THP-1 cells were stimulated with HKLM (10⁸ cells/ml), poly(I:C) (25 mg/ml), flagellin (10⁻⁵ g/ml), loxoribine (1 mM), or ODN2006 (5 mM) instead of LPS. To investigate the effects of immunosuppressants, cells were treated with 10⁻⁵ M of PSL, MPSL, DEX, BDP, MTX, 6MP, CYA, or TAC together with/without LPS (10⁻⁵ g/ml) for 12 h. Dose dependency was also examined in 10⁻⁹–10⁻⁴ M of PSL, MPSL, DEX, and BDP.

Quantitative Real-Time RT-PCR Analysis

Primer sets used were synthesized at Invitrogen (Carlsbad, CA). The primer sets were: PS-PLA₁ (sense, 5'-

CCTTCCTTAGCAGTAACATCACC-3'; antisense, 5'-CAAACCTCGGTTGGAAGACTGAA -3'), group IVA cytosolic PLA₂ (cPLA₂ α) (sense, 5'-ACTCCAGATCCCTATGTGGAAC-3'; antisense, 5'-GCTGTCCCTAGAGTTTCATCCAT-3'), tumor necrosis factor- α (TNF- α) (sense, 5'-ATCTACTCCCAGGTCCTCTTCAA-3'; antisense, 5'-GCAATGATCCCAAAGTAGACCT-3'), β -glucuronidase (GUS) (sense, 5'-TGATGGAAGAAGTGGT GCGTAG-3'; antisense, 5'-AGTTGCTCACAAAGGT CACAGG-3'), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (sense, 5'-GCCAAGGTCATCCATGACAACT-3'; antisense, 5'-GAGGGGCCATCCACAGT CTT-3').

Total RNA was extracted by Isogen (Nippon Gene, Tokyo, Japan) into TE according to the manufacturer's instructions. Single-strand cDNA was synthesized with SuperScript™ III Reverse Transcriptase and real-time PCR was performed with a dsDNA binding dye, SYBR Green I, using SuperScript™ III Platinum® Two-Step qPCR Kit with SYBR Green (Invitrogen, Carlsbad, CA). Fluorescence was detected with Mx3000P system (Stratagene, La Jolla, CA) and analyzed with MxPro software (Stratagene). The expression levels of mRNA for each gene were normalized by that of GUS.

All data are presented as the mean \pm SD. Nonrepeated measures ANOVA followed by Dunnett's test was used to assess any significant differences between the absolute values. Significance was set at $p < 0.05$.

RESULTS

PS-PLA₁ mRNA Expression in Human THP-1-Derived Macrophages

The PS-PLA₁ mRNA expression was compared with the expression of TNF- α mRNA (positive control for TLR response) in the THP-1-derived macrophages after treating the cells with several TLR ligands for 1 and 12 h. Among the TLR ligands [HKLM, poly(I:C), LPS, flagellin, loxoribine, and ODN2006 for TLR2, TLR3, TLR4, TLR5, TLR7, and TLR9, respectively], LPS induced PS-PLA₁ expression at 12 h after treatment, while no other ligands exhibited such induction (data not shown). On the other hand, TNF- α mRNA was induced when the cells were treated with HKLM, LPS, and flagellin for 1 h (data not shown).

LPS treatment increased PS-PLA₁ mRNA expression on THP-1-derived macrophages 800-fold, which was four times greater than that of cPLA₂ α (Fig. 1). The upregulation of PS-PLA₁ mRNA was LPS dose dependent at the concentration of 10⁻⁹–10⁻⁵ g/ml (data not shown). The time course profiles of mRNA expression after LPS stimulation was different between PS-PLA₁ and TNF- α . The maximum mRNA expression for PS-PLA₁ and TNF- α was at 12 and 1 h after LPS stimulation, respectively (Fig. 1).

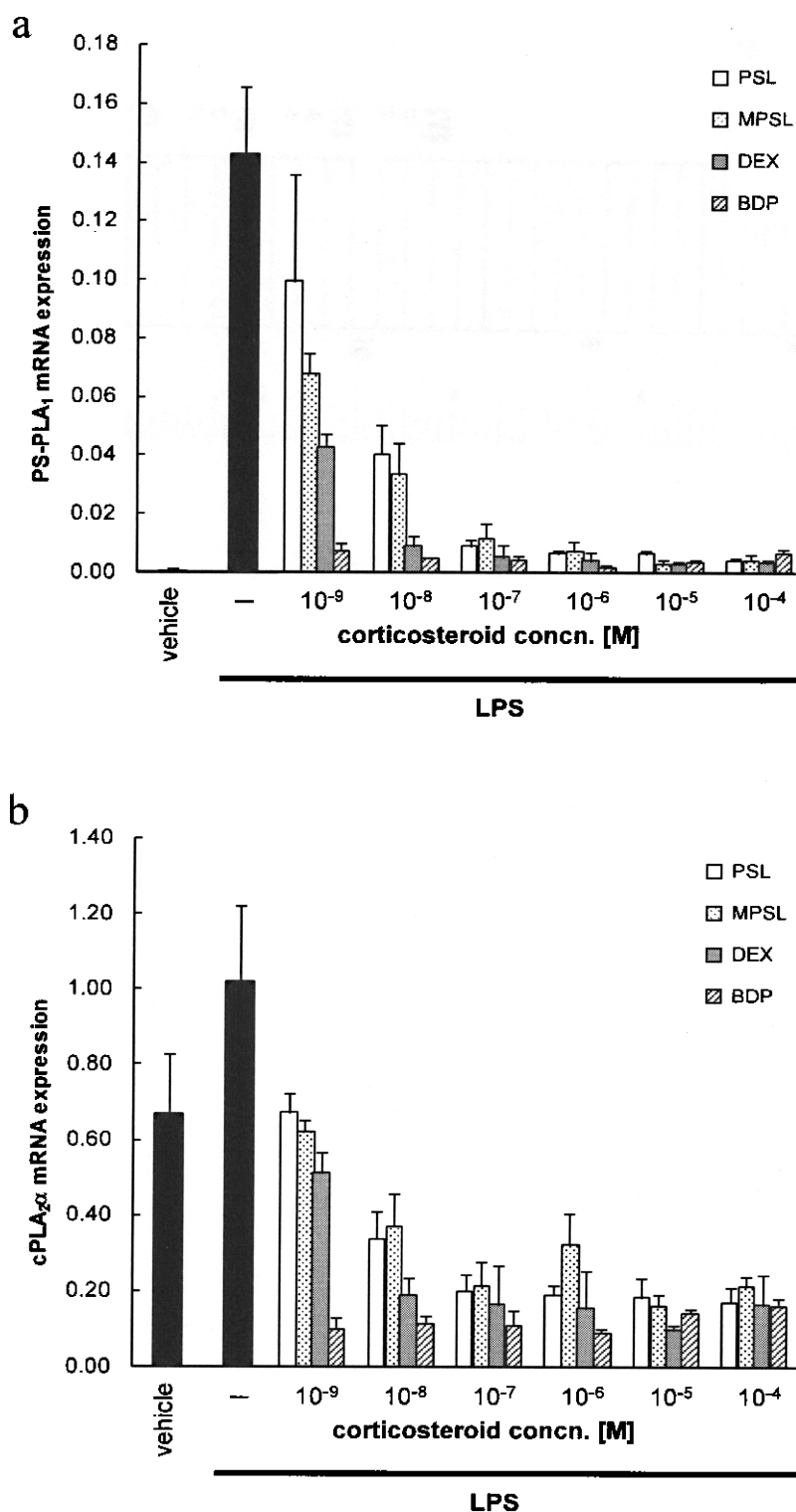


Figure 2. Dose-dependent inhibition of the mRNA expression for PS-PLA₁ and cPLA₂α by corticosteroids. Differentiated THP-1 cells were incubated for 12 h at different concentrations of PSL (open bars), MPSL (dotted bars), DEX (shaded bars), or BDP (hatched bars) in the presence of 10⁻⁵ g/ml of LPS. The relative mRNA levels of PS-PLA₁ (a) and cPLA₂α (b) to GUS were determined, respectively, by quantitative real-time RT-PCR. The results are shown as the mean ± SD of three independent experiments.

Effects of Immunosuppressive Agents on PS-PLA₁ mRNA Expression

Among the immunosuppressive agents, corticosteroids (PSL, MPSL, DEX, and BDP) showed marked inhibition of PS-PLA₁ mRNA expression, while other agents, MTX, CYA, TAC, 6MP and MPA, showed weak or little effects (Table 1). The inhibitory effects of corticosteroids on PS-PLA₁ mRNA expression were dose dependent (Fig. 2). The half-maximal inhibitory concentration (IC₅₀) for PSL was 3.0 nM (Fig. 2). The inhibitory effects of corticosteroids on mRNA expression for cPLA₂α were similar to those for PS-PLA₁ (Fig. 2). Other agents inhibited cPLA₂α mRNA expression, although the inhibitory effects were weaker than that for corticosteroids (Table 1).

DISCUSSION

The present results revealed the expression of PS-PLA₁ mRNA in THP-1-derived macrophages to be activated by treating the cells with LPS, a typical TLR4 ligand, but not with other TLR ligands. The induction of PS-PLA₁ is also observed in rats after the administration of LPS (4). These findings suggest that PS-PLA₁ mRNA is specifically activated via the TLR4 signaling pathways both in vitro and in vivo. On the other hand, cPLA₂α mRNA was expressed on THP-1-derived macrophages in nature and it was little affected by LPS treatment. This means that these two phospholipases play different roles in the immune response with regard to TLR4 activation.

TLR4, a well-known receptor for LPS, interacts with a variety of other ligands including heat shock proteins (HSPs), and it rapidly activates the innate immune system (9,17). The current findings suggest that innate immune events play a critical role in allograft rejection as well as host defense. In the initial allograft injury induced by reactive oxygen species, which are generated during allograft ischemia-reperfusion in implantation (10,14,22), the upregulated HSPs activate TLR4-bearing vascular cells, thus contributing to developing chronic rejection (9). The role of TLR4 in the development of chronic rejection has been also observed in rat recipients of cardiac transplantation (13). Therefore, the PS-PLA₁ mRNA expression via TLR4 specific activation is consistent with the previous finding that PS-PLA₁ is one of the most upregulated expression genes in a rat chronic rejection model, thus suggesting that it can also be possible biomarker for rejection episodes.

Another finding in the present study was that the PS-PLA₁ mRNA expression was strongly inhibited by corticosteroids among the several known immunosuppressive agents (Table 1). cPLA₂α, a key enzyme for prostaglandin biosynthesis (3,23), is strongly inhibited by corticosteroids in their anti-inflammatory action (5). The mag-

nitude of the inhibitory effects for PS-PLA₁ mRNA expression was almost the same as those for cPLA₂α (Fig. 2). This means that corticosteroids may exert different immunosuppressive effects on the status of PS-PLA₁ expression via TLR4 activation. High-dose corticosteroids for chronic rejection (8) may therefore be a reasonable therapeutic strategy if PS-PLA₁ plays an important role. Further study is thus required to confirm this speculation.

In conclusion, PS-PLA₁ mRNA was specifically expressed via TLR4 activation in THP-1-derived macrophages. This expression was inhibited by corticosteroids, but not by other immunosuppressive agents. The clinical application of this potential treatment modality in allograft immunity therefore requires further investigation.

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